

Tumorigenesis and Neoplastic Progression

Overexpression of Heme Oxygenase-1 in Murine Melanoma

Increased Proliferation and Viability of Tumor Cells, Decreased Survival of Mice

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Heme oxygenase-1 (HO-1), a cytoprotective enzyme, can be induced in tumors in response to anti-cancer therapies. We investigated the role of HO-1 in B16(F10), S91, and Sk-mel188 melanoma cells. Overexpression of HO-1 after transduction with adenoviral vectors increased cell proliferation, resistance to oxidative stress generated by H₂O₂, and angiogenic potential as determined by induction of endothelial cell divisions. Likewise, cells stably transfected with HO-1 cDNA (B16-HO-1) showed higher proliferation, stress resistance, and angiogenic activity than the wild-type line (B16-WT). HO-1 overexpression in tumors significantly shortened survival of mice after subcutaneous injection of cancer cells (38 and 22 days for B16-WT and B16-HO-1, respectively; *P* = 0.017). This also resulted in development of more packed tumors, with more melanoma cells, and reduced inflammatory edemas. Mice injected with B16-HO-1 had

lower levels of tumor necrosis factor and higher serum concentrations of its soluble receptor tumor necrosis factor-RI, whereas tumors overexpressing HO-1 displayed augmented vascularization and stronger production of vascular endothelial growth factor. Finally, B16-HO-1 cells injected intravenously formed more metastases in lungs. Thus, HO-1 overexpression increased viability, proliferation, and angiogenic potential of melanoma cells, augmented metastasis, and decreased survival of tumor-bearing mice, suggesting that induction of HO-1 may be detrimental in anti-cancer therapy of melanoma. (Am J Pathol 2006, 169:2181–2198; DOI: 10.2353/ajpath.2006.051365)

Heme oxygenases (HOs) catalyze the oxidation of heme to the biologically active products carbon monoxide (CO), biliverdin, and ferrous iron. Two distinct variants of HOs have been described in humans and rodents, each encoded by a different gene: *HO-2*, which is constitutively expressed, and *HO-1*, which is potently induced in many cell types by heme, inflammatory cytokines, and oxidative stress-related factors.¹

Several lines of evidence have shown that HO-1, a 32-kd microsomal enzyme, participates in maintaining the cellular homeostasis and plays an important protective role in the tissues due to reducing oxidative injury and attenuating the inflammatory response. Pharmacological activation or genetic augmentation of HO-1 can

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provide cellular protection in cultured cells and in several animal models of brain, heart, kidney, lung, and liver failures.^{2–6} In addition, HO-1 can protect different cell types from apoptosis induced by a wide spectrum of stimuli, such as serum deprivation, staurosporine, etoposide, tumor necrosis factor (TNF), cyclohexamide, cisplatin, interleukin-1 α , and interferon- γ .¹ Accordingly, it has been demonstrated that inhibition of HO-1 activity can make vascular smooth muscle cells, endothelial cells, and fibroblasts more susceptible to oxidative stress and apoptotic death.^{7–9}

HO-1 is also involved in regulation of cell cycle, although these effects are tissue-dependent. In keratinocytes^{10,11} and in vascular endothelium,¹² induction of HO-1 has been shown to stimulate cell cycle progression, whereas in vascular smooth muscle cells it can attenuate proliferation.¹³ However, only a few, and still not consistent, results have been published on the role of HO-1 in regulation of cell cycle in the cancer cells. Namely, in breast carcinoma HO-1 possibly inhibits proliferation,¹⁴ whereas in pancreatic tumors it may promote cell division.¹⁵ Nothing is known, however, about the effect of HO-1 in regulation of cell cycles in other types of cancers, including melanoma.

Finally, it has been demonstrated that activation of HO-1 may increase angiogenesis, a formation of new blood vessels from pre-existing ones.¹⁶ Angiogenesis is the fundamental process contributing to tumor growth and metastasis.¹⁷ Its augmentation can be associated with up-regulation of prominent proangiogenic protein, vascular endothelial growth factor (VEGF), as shown in vascular smooth muscle cells, macrophages, keratinocytes, or microvascular endothelium.^{18–21} In addition, expression of HO-1 improves the response of endothelial cells to exogenously delivered VEGF.¹² Noteworthy, the proangiogenic potential of HO-1 has been confirmed *in vivo*, eg, in the rat hind limb ischemia model, in which overexpression of HO-1 after delivery of cDNA with adenoviral vectors strongly increased synthesis of VEGF and improved neovascularization of the ischemic muscle.²² Some reports also suggest a correlation between angiogenesis and HO-1 expression in tumors, namely in glioma and pancreatic carcinoma.^{16,23}

Most studies concerning the role of HO-1 have been performed in the cardiovascular system. It should be noted, however, that elevated expression of HO-1 has also been detected in various neoplastic cells, including human adenocarcinoma, hepatoma, glioblastoma, squamous carcinoma, prostatic cancers, Kaposi sarcoma, and melanoma.^{24–30} Importantly, its expression in tumors can be potently induced in response to radio-, chemo-, or photodynamic therapies.^{15,31,32} Nevertheless, the knowledge about the influence of HO-1 induction on progression of tumors is limited. Specifically, there are no data concerning the role of HO-1 in melanoma, the type of tumor relatively resistant to chemotherapeutic treatment. Therefore, our aim was to determine the effect of HO-1 overexpression on proliferation, viability, and angiogenic potential of murine and human melanoma cells.

Materials and Methods

Reagents

Carboxymethylcellulose, Triton X-100, phenylmethyl sulfonyl fluoride, leupeptin, aprotinin, bicinchoninic acid protein assay kit, BCIP/NBT, eosin, hematoxylin, HEPES, and G418 were purchased from Sigma (Poznan, Poland). Tin protoporphyrin-IX (SnPPIX) was from Porphyrin Products (Logan, UT). The total RNA extraction kit, reverse transcription system, PCR core system, agarose, and luciferase assay reagents were obtained from Promega (Madison, WI). RNA isolation kit (for microarray analysis), Maxiprep QIAfilter EndoFree plasmid isolation kit, RNeasy mini kit, and SuperFect transfection reagent were purchased from Qiagen (Hilden, Germany). Blocking antibodies against VEGF and enzyme-linked immunosorbent assay (ELISA) kits for mouse VEGF, TNF, and soluble TNF receptor-I (sTNF-RI) were obtained from R&D Systems (Minneapolis, MN). BrdU incorporation ELISA was obtained from Roche Diagnostics (Mannheim, Germany). ELISA kit for rat HO-1 was obtained from Stressgen Biotechnologies (Victoria, BC, Canada). Nitrocellulose membrane HybondECL was procured from Amersham Pharmacia Biotech (Boston, MA). Rabbit polyclonal antibodies recognizing HO-1 and goat anti-rabbit monoclonal antibodies conjugated with biotin were purchased from Stressgen Biotechnologies, alkaline phosphatase-conjugated streptavidin from DAKO (Carpinteria, CA), CD31 anti-mouse goat polyclonal antibody from Santa Cruz Biotechnologies (Santa Cruz, CA), and peroxidase-conjugated donkey anti-goat antibody from Jackson ImmunoResearch Laboratories (Aurora, OH). Mouse cytokine antibody array was delivered from Ray-Biotech (Norcross, CA). Expression plasmid pcDNA3.1⁺ was obtained from Invitrogen (San Diego, CA), and heparin and endothelial cell growth supplement from Upstate Biotechnology (Charlottesville, VA). Adeno-X adenoviral expression system and Adeno-X rapid titer ELISA kit were purchased from Clontech (Mountain View, CA). All other reagents were procured from Gibco BRL (Paisley, UK).

Cell Culture

S91 and B16(F10) murine melanoma cell lines and human melanoma Sk-mel188 cell line were maintained in RPMI 1640 medium containing 10% fetal bovine serum, glutamax (2 mmol/L), penicillin (100 U/ml), and streptomycin (0.1 μ g/ml) at 37°C, in a humidified atmosphere with 5% CO₂. B16(F10) cells stably overexpressing HO-1 (B16-HO-1) were cultured in the same medium supplemented additionally with G418 (0.4 mg/ml) as a selective agent. S91 and Sk-mel188 cells were kindly provided by Dr. Maja Grabacka, Krakow, Poland.

Some experiments were performed on melanoma cells cultured under hypoxic conditions in a modular incubator chamber (Billups-Rothenberg Inc., Del Mar, CA). Cells were treated according to experimental protocols and then placed in the hypoxia chamber, which was flushed for 20 minutes with a gas mixture (1% O₂, 5% CO₂, and 94% N₂). Then chamber was tightly sealed and placed at

37°C in a standard incubator for 24 hours. Human umbilical vein endothelial cells (HUVECs) were freshly isolated from human umbilical veins of newborn babies by collagenase digestion. HUVECs were grown in M199 medium with 20% fetal bovine serum supplemented with 20 mmol/L HEPES, 30 ng/ml heparin/endothelial cell growth supplement, penicillin (100 U/ml), and streptomycin (0.1 µg/ml). Cells of passages three to five were used in experiments.

Preparation of Adenoviruses

Adenoviral vectors containing rat HO-1 cDNA (Ad-HO-1) were kindly gifted by Dr. Gisa Tiegs (Erlangen, Germany). Control vectors harboring β -galactosidase cDNA (Ad- β gal) were produced using the Adeno-X system. Both vectors were propagated in 293 cells (six passages) and then titered by detection of hexon protein with Adeno-X rapid titer ELISA kit according to the vendor's protocols. Melanoma cells were transduced with multiplicity of infection (MOI) of 10 virus particles/cell 48 hours before experiments.

Preparation of Plasmids

Rat HO-1 cDNA (a kind gift of Dr. Mahin Maines, Rochester, NY) was subcloned to pcDNA3.1⁺ expression plasmid as described previously.¹⁸ Reporter plasmid pGL2-VEGF was kindly provided by Dr. Hideo Kimura (Chiba, Japan). It contains the luciferase reporter gene driven by a full sequence of human VEGF promoter. Plasmids were isolated from bacteria culture using End-free plasmid isolation kit. The integrity of DNA was determined by electrophoresis in 1% agarose gel.

Transfection of Cells with HO-1 and VEGF Expression Plasmids

Transfection was performed using SuperFect transfection reagent according to the manufacturer's instructions. In brief, cells were grown in a 24-well plate to reach the confluence of 70%. For each well, 0.5 µg of plasmid DNA was mixed with 2.5 µl of SuperFect and 30 µl of empty RPMI 1640 medium. After a 10-minute incubation at room temperature, 350 µl of complete medium was added. Cells were washed twice with phosphate-buffered saline (PBS) and exposed to the transfection mixture for 5 hours. At the end of incubation period, medium was replaced with a fresh one for an additional 48 hours. Then, cells were cultured for 3 weeks in a selecting medium containing 1 mg/ml G418. Successful stable transfection was confirmed by polymerase chain reaction (PCR), reverse transcriptase (RT)-PCR, and Western blotting. Transfected cells were maintained in medium supplemented with lower concentration of G418 (0.4 mg/ml). Experiments were performed in medium without this antibiotic.

Luciferase Assay

Luciferase activity assay was performed in the cellular extracts according to the vendor's protocol. Lysates were

prepared 48 hours after transfection of melanoma cells with pGL2-VEGF plasmid. Results were normalized to total protein concentration.

Measurement of Protein Level

Total protein concentrations were determined using a bicinchoninic acid protein assay kit, according to the manufacturer's instructions.

RNA Isolation

Total RNA was isolated by a phenol-chloroform extraction. In brief, cells cultured in a 24-well plate were washed with PBS, overlaid with 300 µl of acid guanidinium thiocyanate, and mixed with 300 µl of phenol/chloroform/isoamyl alcohol mixture. The harvested lysate was vortexed, incubated on ice for 30 minutes, and centrifuged (30 minutes, 10,000 × *g*, 4°C). Then, an upper aqueous phase was collected and subjected to ethanol precipitation. RNA pellet was dissolved in nuclease-free water.

RT-PCR

Reverse transcription was performed on 1 to 2 µg of total RNA for 1 hour at 42°C using oligo-dT primers and AMV reverse transcriptase. PCR amplification with *Taq* polymerase was performed for 30 cycles using the following protocol: 95°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute (for HO-1 and GAPDH), or 95°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute (for VEGF). The following primers were used: 5'-GTGGAGACGCTTACGTAGTGC-3' and 5'-CTTTCAGAAGGGTCAGGTGTCC-3' for HO-1, 5'-CTGCTCTCTTGGGTGCACTG-3' and 5'-CACCGCCTTGGCTTGTCACAT-3' for VEGF, and 5'-CGTATTGGGCGCCTGGTCA-CC-3' and 5'-CTTTCAGAAGGGTCAGGTGTCC-3' for the housekeeping gene GAPDH. PCR products were analyzed by agarose gel electrophoresis.

Western Blotting

Expression of HO-1 at the protein level was checked in the melanoma cells cultured in six-well plates. Cells were lysed in ice-cold lysis buffer (1% Triton X-100, 1 µg/ml phenylmethyl sulfonyl fluoride, 1 µg/ml leupeptin, and 1 µg/ml aprotinin) and centrifuged for 20 minutes, 8000 × *g*, at 4°C. Clear supernatants were collected, and protein samples (15 µg/well) were subjected to electrophoresis in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose membrane HybondECL. Then, membranes were probed with polyclonal antibodies against HO-1 and treated with biotin-conjugated secondary antibodies (diluted 1:2500 in Tris-buffered saline with 3% albumin). Blots were labeled with alkaline phosphatase-conjugated streptavidin (diluted 1:5000 in Tris-buffered saline) and visualized using BCIP/NBT blue liquid substrate.

Trypan Blue Exclusion Assay

Cell viability was assessed by staining with trypan blue (0.2%) for 30 seconds and counting cells in three randomly selected fields of a Burk chamber.

Measurement of Cell Proliferation

Proliferation of melanoma cells or HUVECs was measured in cells cultured in 96-well plates (seeded at the density of 3×10^3 per well) using BrdU incorporation assay. B16-WT or B16-HO-1 cells were cultured in complete medium for 18 hours, BrdU was added for 2 hours, and BrdU incorporation was measured according to the vendor's protocol. To measure angiogenic properties of melanoma, 3×10^3 HUVECs were seeded in the mixture of 50 μ l of incomplete M199 medium (10% fetal calf serum, without endothelial cell growth supplement) and 50 μ l of conditioned medium collected from B16-WT or B16-HO-1. BrdU incorporation assay was performed after 48 hours.

Spheroid Culture Assay

Experiments were performed according to the procedure described previously¹² in medium with 10% fetal calf serum, but without endothelial cell growth supplement. In brief, to generate endothelial cell spheroids, 750 cells were suspended in culture medium containing 0.25% (w/v) carboxymethylcellulose and seeded in nonadherent round-bottom 96-well plates. During 24 hours, all suspended cells contributed to the formation of a single spheroid. These spheroids were harvested and embedded in collagen gels, prepared by mixing acidic collagen extract of rat tails with $10\times$ RPMI medium and 0.1 N NaOH. Media harvested from melanoma cells (200 μ l/well) were added on top of the gel. Under such conditions, spheroids formed capillary-like sprouts, which were inspected and measured after 24 hours using a digitized imaging system connected to an inverted microscope.

Inoculation of Tumor Cells and in Vivo Assessment of Tumor Growth

All procedures were performed according to the local bioethic commission protocols. In the first set of experiments C57BL/6 mice were divided into two groups (10 animals per group), and 100 μ l of saline containing 2×10^5 B16-WT or B16-HO-1 cells were inoculated subcutaneously. Using a caliper, the tumor diameters were measured every day, and tumor volumes were determined using the following formula: $V = D \times d^2 \times 0.52$ (V , tumor volume; D , the biggest dimension; d , the smallest dimension).

In the second set of experiments C57BL/6 mice were injected with B16-WT or B16-HO-1 cells into the tail vein (2×10^5 cells in 100 μ l of saline, 10 mice per group at each time point). Animals were sacrificed on days 7, 14, and 21 after inoculation, when the lungs were removed and fixed in Bouin solution for 24 hours. Then the number of metastases was calculated by two researchers in a blinded manner.

Screening for Inflammatory Cytokines in the Blood

Concentrations of 22 cytokines (RayBio Mouse Cytokine Array I) were assessed using mouse cytokine antibody array in 1 ml of 10-fold diluted serum per array. The obtained results were then confirmed with appropriate ELISAs.

Preparation of Tissue Lysates

Tumor specimens were homogenized on dry ice and suspended in ice-cold PBS containing 1% Triton X-100 and protease inhibitors (1 μ g/ml phenylmethyl sulfonyl fluoride, 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin). Then samples were incubated for 30 minutes on ice and centrifuged ($21,000 \times g$, 10 minutes, 4°C), and clear supernatant was collected. Total protein concentration was determined by the bicinchoninic acid protein assay kit.

Measurement of Transgene HO-1 Expression in Tumors

Concentration of rat HO-1 protein in tumor lysates was determined using a rat HO-1 ELISA according to the vendor's instructions.

Measurement of VEGF, TNF, and sTNF-RI Protein Levels

Concentrations of these proteins in the culture media, blood serum, and tumor lysates were measured using colorimetric sandwich ELISA according to the vendor's instructions.

Immunohistochemistry

Tissue specimens were collected from three levels of tumors. Frozen tissue sections (6 μ m in thickness) were fixed with ice-cold acetone. Endothelial cells were detected using CD31 goat anti-mouse polyclonal antibody (diluted 1:125) and visualized by peroxidase-conjugated donkey anti-goat antibody (diluted 1:500). Tissues were counterstained with hematoxylin. To determine the capillary density, all vessels visible in the specimen were counted under a $\times 40$ objective and are expressed as a mean number of capillaries per field. The whole surface of the specimen was analyzed. In parallel, hematoxylin and eosin (H&E) staining was performed to visualize necrosis in tumor tissue. Then, the necrotic areas from each specimen were examined under a $\times 20$ objective and shown as a percentage of total area.

Microarray Analysis

RNA was isolated using RNeasy mini kit, according to the vendor's instructions, from three different cell batches for both control and HO-1-overexpressing samples. Microarrays were prepared in-house (INSERM U533-Ouest G  n  pole, Nantes, France) using 50-mer oligonucleotide probes (MWG Biotech, Roissy, France), obtained from the

MWG Biotech human genome-wide microarray collection and tested for specificity. The probes were diluted in spotting buffer (25 $\mu\text{mol/L}$ in buffer A 1 \times ; MWG Biotech) and spotted onto epoxy-silane-coated glass slides (Slide E; Schott Nexterion, Jena, Germany) using the Lucidea array spotter from Amersham. Immediately after spotting, the slides were incubated at 42°C and 55% humidity for 15 hours. Slides were washed once in 0.2% sodium dodecyl sulfate and twice in H_2O and then incubated for 20 minutes in H_2O at 50°C, dried by centrifugation ($40 \times g$, 3 minutes, 20°C), and stored at 20°C.

The 6864 genes that were represented on the microarray were selected for involvement in different cancers. Each gene was spotted in triplicate on the microarrays. Cy3- and Cy5-labeled (amplified) aRNA was prepared using the Amino Allyl MessageAmpII aRNA amplification kit (Ambion, Austin, TX). The reference sample consisted of a pool of an equal amount of control tRNA from each cell culture without plasmid and was labeled with Cy5. For each of six different cultures of the same cells with the HO-1 plasmid and each control tRNA of three different cultures of the same cells without the HO-1 plasmid, two replicate tRNA extractions were labeled individually with Cy3. Each Cy3-labeled sample was mixed with an equal amount of Cy5-labeled reference sample, preincubated with human Cot-I DNA (Gibco BRL), yeast tRNA, and polyA RNA, and hybridized to the microarrays. For each hybridization, a different Cy5-labeled sample was used. Hybridized arrays were scanned at 10 μm /pixel resolution by fluorescence confocal microscopy (Scanarray 3000; GSI-Lumonics, Billerica, MA). Signal intensities were extracted with Genepix Pro 5.0 image analysis software (Axon Instruments, Sunnyvale, CA). Data were retained based on concordance between the measurements of different types of replicates: biological replicates, technical replicates, and combined biological and technical replicates.

Fluorescence-Activated Cell Sorting Analysis

Tumors were cut into small pieces and rubbed through a sieve. The obtained cell suspensions were stained with directly conjugated rat anti-mouse CD45-RPE monoclonal antibody or isotype-matched RPE rat immunoglobulins (DAKO). CD45-positives were gated and analyzed using fetal calf serum/standard saline citrate morphological parameters by means of FACScan flow cytometer and Cell Quest software (BD Biosciences). Each analysis included 10,000 events.

Statistical Analysis

In vitro experiments were performed in duplicates or triplicates and repeated at least two times. *In vivo* experiments were performed on 10 animals per group. Results are presented as mean \pm SD. Statistical significance was determined using Student's *t*-test or analysis of variance followed by Tukey test for comparison of two and more samples, respectively. Survival curves were compared by Kaplan-Meier Wilcoxon analysis.

Results

Effect of HO-1 Overexpression in Melanoma Cell Lines

Overexpression of HO-1 was produced by transduction of cells with adenoviral vectors containing rat HO-1 cDNA under control of the CMV promoter (Ad-HO-1, 10 MOI/cell). Control cells were transduced with the same dose of vectors harboring β -galactosidase (Ad- β gal). Transduction efficacy ($\sim 40\%$) and overexpression of HO-1 were confirmed by *in situ* β -galactosidase staining and RT-PCR, respectively (not shown). We compared the effects of transduction in murine [B16(F10) and S91] and human (Sk-mel188) melanoma cell lines. In all melanomas studied, overexpression of HO-1 resulted in increased proliferation, as measured by BrdU incorporation assay (Figure 1, A–C). In B16(F10) and Sk-mel188 cells, BrdU incorporation was $\sim 30\%$ higher than in their counterparts transduced with Ad- β gal. In S91, this increase was weaker and did not reach statistical significance.

Next, we compared sensitivity of melanoma cells to oxidative stress induced by 4 hours of exposure to H_2O_2 (25 to 800 $\mu\text{mol/L}$) (Figure 1, D–F). In cultures of B16(F10), cell viability was reduced already at a 50 $\mu\text{mol/L}$ concentration of H_2O_2 , decreasing to $\sim 20\%$ at the dose of 800 $\mu\text{mol/L}$. Lines S91 and Sk-mel188 were much more resistant, and even in the presence of 400 $\mu\text{mol/L}$ H_2O_2 , their viability was $\sim 80\%$. Nevertheless, in all cell lines overexpression of HO-1 had a protective effect, very strong in B16(F10) and visible only at the highest doses of H_2O_2 in S91 and Sk-mel188.

Finally, we investigated the angiogenic potential of melanoma cells overexpressing HO-1. To this aim, we measured proliferation of HUVECs incubated for 48 hours in the media harvested from control melanoma and that transduced with Ad- β gal or Ad-HO-1 (Figure 1, G–I). In all cases, media from melanoma cell cultures significantly augmented proliferation of HUVECs. This mitogenic effect was stronger ($\sim 30\%$ for each cell line) if cancer cells overexpressed HO-1.

Thus, results obtained by transduction of three melanoma cell lines with Ad-HO-1 indicate that a high level of HO-1 expression leads to increased cell proliferation, improved viability under oxidative stress, and stronger angiogenic potential (Figure 1). All cell lines behaved similarly when proliferation or angiogenic activity was measured, whereas sensitivity to oxidative stress and, in consequence, the importance of protective effect of HO-1 were cell line-dependent.

Stable Overexpression of HO-1 in B16-HO-1 Cells

To avoid side effects of adenoviral transduction and to obtain melanomas with a high expression of HO-1 in all cells, we decided to prepare the B16(F10) cell line stably transfected with pcDNA3.1 plasmid, containing the rat HO-1 cDNA under control of the CMV promoter. Resulting

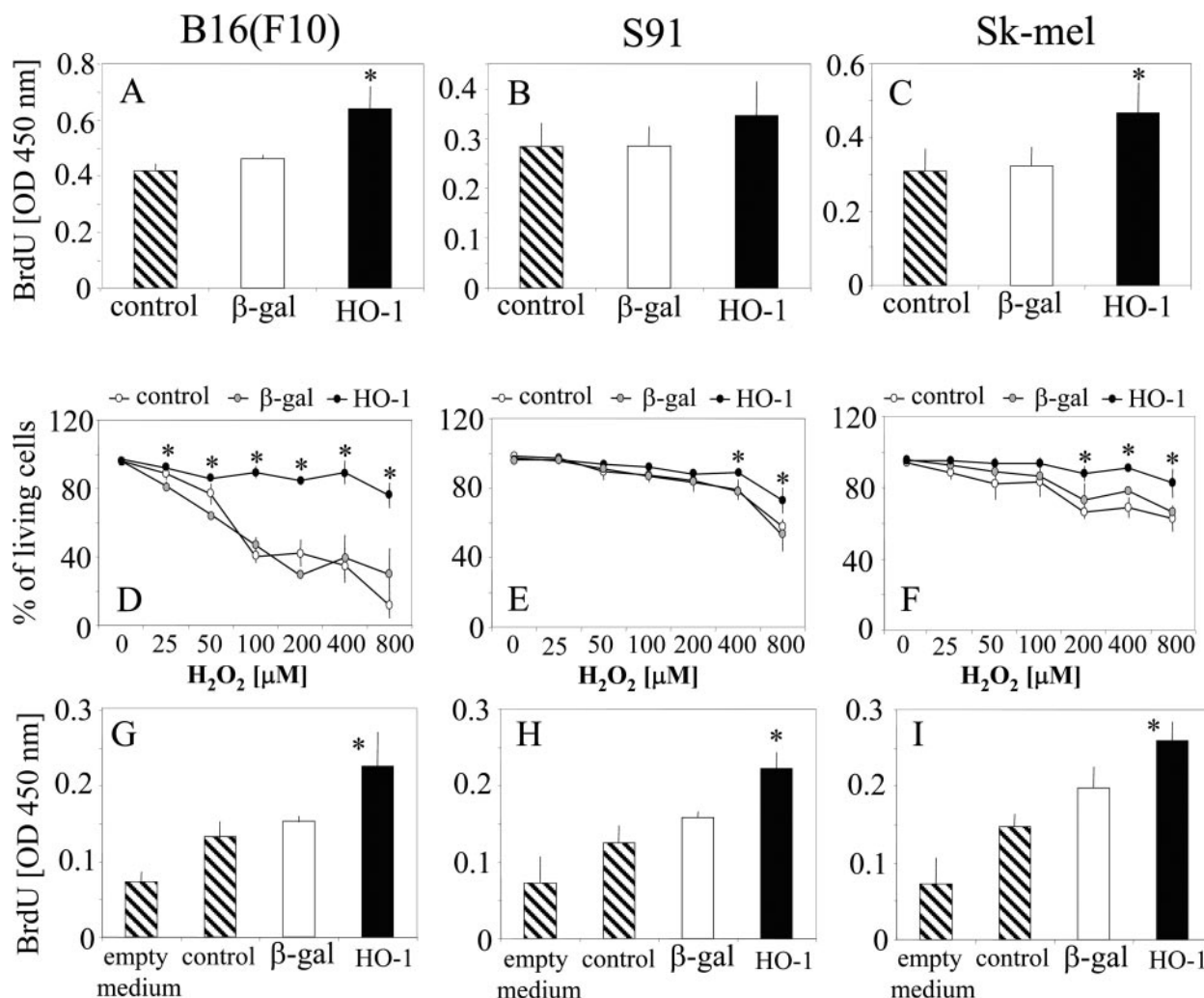


Figure 1. Effect of transduction of B16(F10) (A, D, G), S91 (B, E, H), and Sk-mel188 (C, F, I) melanoma cell lines with adenoviral vectors (10 MOI/cell) harboring HO-1 or β -gal cDNAs. Control cells were not transduced. **A–C:** Effect on melanoma proliferation. Cells were seeded in a 96-well plate (3000/well) and incubated for 18 hours in complete medium. Proliferation was measured by BrdU incorporation assay. **D–F:** Effect on viability of cells cultured in complete medium and exposed to H_2O_2 (25 to 800 μ M/L) for 4 hours. Viability was measured by trypan blue exclusion assay. **G–I:** Effect of nonconditioned (empty) medium and conditioned media harvested from nontransduced (control) or transduced melanoma cell lines on proliferation of HUVECs. Proliferation was measured using BrdU incorporation assay 48 hours after stimulation. Data show one of three similar experiments performed in triplicates. Each bar or point represents mean \pm SD of triplicate. * $P < 0.05$ in comparison with β -gal-transduced cells.

transfectants (B16-HO1) were selected in the presence of G418 antibiotic. To confirm the stable transfection, the genomic DNA was isolated from B16-HO-1 cells and subjected to PCR with primers adjusted to different HO-1 exons. This procedure allowed for amplification of transgene HO-1 cDNA but not the endogenous HO-1 gene. As seen in Figure 2A, HO-1 cDNA was detected in the B16-HO-1 cell line but not in the nontransfected B16-WT melanomas.

The intensities of HO-1 expression in the control and transfected cells were compared at the mRNA level (using RT-PCR with primers recognizing rat and mouse HO-1) and at the protein level (using Western blotting with antibodies against rat and mouse HO-1). Both assays showed that HO-1 can be detected in control B16-WT cells, but much stronger expression was found in B16-HO-1 cell line (Figure 2, B and C). This up-regulation was, however, within the range typical for stimulation with natural HO-1 inducers, as shown in the Western blot prepared from B16-WT cells incubated for 6 hours with 3

or 10 μ M/L hemin (Figure 2D). Accordingly, microarray analysis confirmed that HO-1 mRNA was fourfold more abundant in B16-HO-1 than that in B16-WT cells.

Effect of HO-1 Overexpression on Cell Proliferation

To investigate the effect of HO-1 overexpression on the proliferation of melanoma, the same numbers of B16-WT or B16-HO-1 cells were seeded in a 96-well plate and cultured in complete medium for 18 hours. As measured by BrdU incorporation assay, overexpression of HO-1 led to an almost 2.5-fold increase in cell proliferation. This effect was attenuated by SnPPiX, a competitive inhibitor of HO-1, supporting the involvement of the HO-1 pathway. It should be noted, however, that only the high concentrations of SnPPiX (30 and 100 μ M/L) were ef-

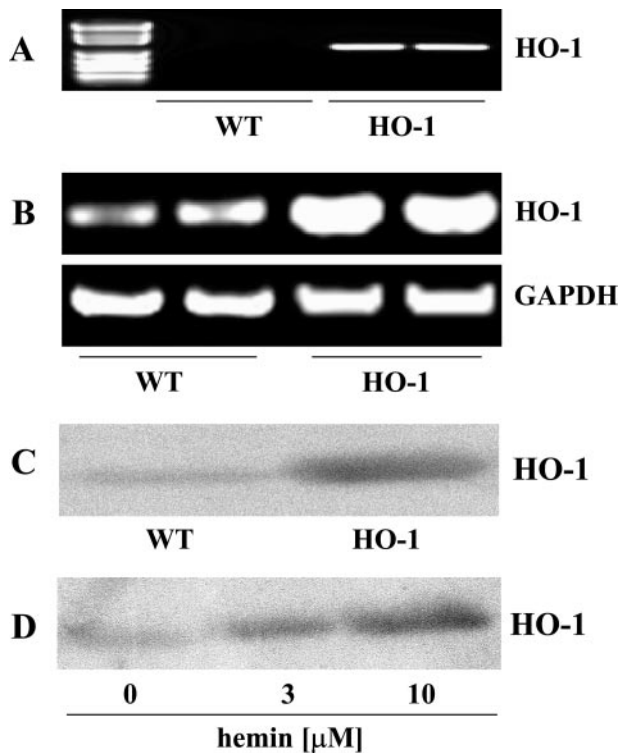


Figure 2. Expression of HO-1 in B16-WT and B16-HO-1 melanoma cell lines cultured *in vitro*. **A:** Electrophoresis of PCR products showing the presence of HO-1 cDNA. **B:** Electrophoresis of RT-PCR products showing the expression of HO-1 mRNA. Primers recognized both rat and murine HO-1. GAPDH was used as a control housekeeping gene. **C:** Western blot showing the expression of HO-1 protein in B16-WT and B16-HO-1 cells (15 μ g of total protein were loaded to each well). **D:** Western blot showing the expression of HO-1 protein in B16-WT cells incubated for 6 hours with hemin (15 μ g of total protein were loaded to each well).

fective (Figure 3), which can result from a high level of HO-1 expression in the transfected cells (Figure 2).

Effect of HO-1 Overexpression on Cell Viability

Our next aim was to determine the influence of stable overexpression of HO-1 on sensitivity of melanoma to

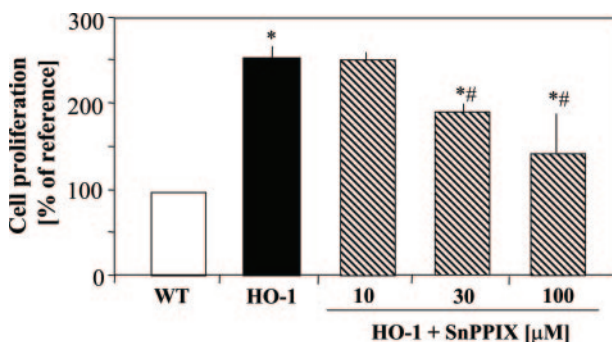


Figure 3. Effect of HO-1 overexpression on proliferation of melanoma cells. B16-WT or B16-HO-1 cells (3000/well) were seeded in a 96-well plate and incubated for 18 hours in complete medium. Some B16-HO-1 cells were additionally supplemented with SnPPiX (10 to 100 μ M), a HO-1 inhibitor. Proliferation was measured by BrdU incorporation assay. Data are presented as a percentage of reference value (reference, B16-WT cells). Each bar represents mean \pm SD of three experiments. * P < 0.05 in comparison with B16-WT; # P < 0.05 in comparison with B16-HO-1 alone.

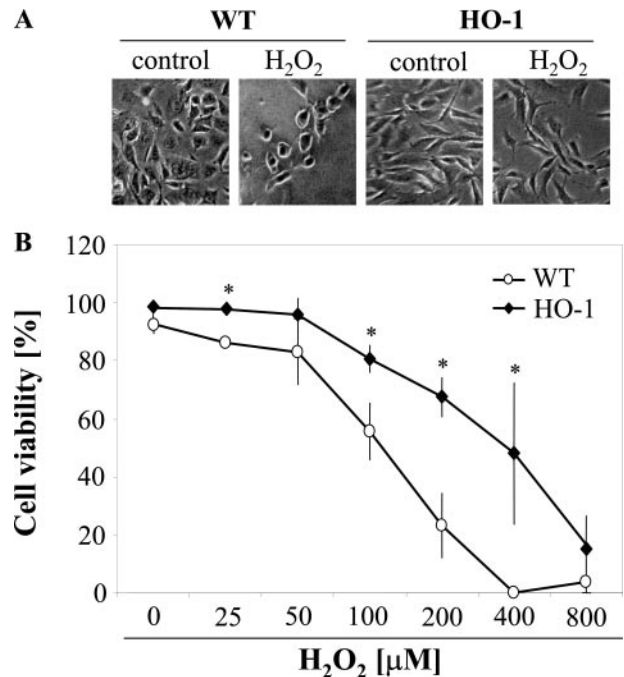


Figure 4. Effect of HO-1 overexpression on viability of melanoma cells. B16-WT and B16-HO-1 cells were cultured in complete medium and exposed to H_2O_2 (25 to 800 μ M/L) for 4 hours. **A:** Representative images showing the appearance of the cells incubated for 4 hours without or with 200 μ M/L H_2O_2 (contrast phase microscopy). **B:** Viability of cells measured by trypan blue exclusion assay. Each point represents mean \pm SD of three experiments. * P < 0.05 in comparison with B16-WT.

oxidative stress. Cultures of B16-WT and B16-HO-1 cells were treated for 4 hours with different concentrations of H_2O_2 (25 to 800 μ M/L). Such a treatment led to changes in cell morphology and concentration-dependent decrease in cell viability (Figure 4). Both B16-WT and B16-HO-1 cells exposed to the oxidative stress shrunk and detached from the plate. However, this effect was much stronger in the wild type than in the HO-1-overexpressing melanoma. For example, in the presence of 200 μ M/L H_2O_2 , most B16-WT cells were rounded, whereas B16-HO-1 cells at the same conditions looked healthy (Figure 4A). Accordingly, the significant differences were found when cell viability was measured using trypan blue exclusion assay. Under the oxidative stress caused by 25 to 400 μ M/L H_2O_2 the B16-HO-1 melanoma survived much better than B16-WT cells. Only the highest dose used (800 μ M/L) was equally toxic to both cell lines (Figure 4B).

Effect of HO-1 Overexpression on Angiogenic Properties of Melanoma Cells

Finally, we decided to investigate the influence of stable overexpression of HO-1 on angiogenic potential of melanoma. Therefore, conditioned media harvested from the confluent melanoma cell cultures were used for stimulation of HUVECs. We found that HUVECs incubated with media collected from B16-WT line proliferated up to five-fold stronger than those cultured with the control, unconditioned media, evidencing the angiogenic properties of

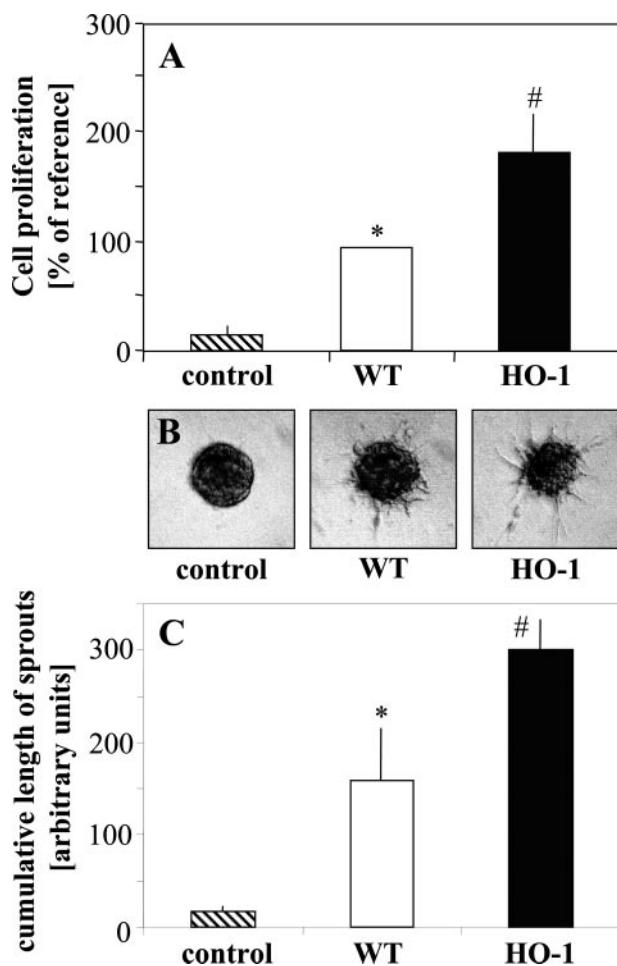


Figure 5. Effect of HO-1 overexpression on proangiogenic properties of melanoma cells. **A:** Effect of control unconditioned medium and conditioned media harvested from B16-WT or B16-HO-1 melanoma cell lines on proliferation of HUVECs. Proliferation was measured using BrdU incorporation assay 48 hours after stimulation. Data are presented as a percentage of reference value (reference, stimulation with media from B16-WT cells). **B:** Representative images showing the effect of control unconditioned medium and conditioned media harvested from B16-WT or B16-HO-1 melanoma cell lines on sprouting of capillaries from endothelial spheroids embedded in the collagen gel. **C:** Cumulative length of sprouts growing from endothelial spheroids. Each bar represents mean \pm SD of three (**A**) or four (**C**) experiments. * $P < 0.05$ in comparison with control; # $P < 0.05$ in comparison with B16-WT.

melanoma cells (Figure 5A). Of importance, such a stimulation of HUVEC proliferation was significantly more potent in response to media collected from B16-HO-1 cultures (up to 10-fold increase when compared with unconditioned control). Furthermore, media harvested from both melanoma cell lines increased the outgrowth of capillaries from the endothelial spheroids embedded in the collagen gels (Figure 5, B and C). As in the case of proliferation, this effect was approximately twofold stronger for B16-HO-1 than B16-WT melanoma cells. Taken together, these results show that high expression of HO-1 increases the angiogenic potential of melanoma.

Production of VEGF in Melanoma Cells

HO-1 is known to increase angiogenesis by up-regulation of VEGF, one of the most important proangiogenic medi-

ators.³³ We found that B16-WT and B16-HO-1 melanoma cells expressed VEGF mRNA and protein, as demonstrated by RT-PCR and ELISA, respectively (Figure 6A). The concentration of VEGF released to the medium after an overnight incubation was relatively high, ranging from 300 to 700 pg/ml. The amount of VEGF released was, however, similar in both cell lines, regardless of the level of HO-1 expression. Of note, the high production of VEGF in melanoma could be further augmented in response to some stimuli, eg, if the cells, used as a positive control, were cultured under hypoxic conditions. Likewise, overexpression of HO-1 did not influence the activity of the VEGF promoter in melanoma cells transfected with pGL2-VEGF plasmid because the luciferase assay gave the same results for B16-WT and B16-HO-1 cell lines (Figure 6B). Again, the transcription driven by VEGF promoter was potently induced when cells were incubated under hypoxic conditions, demonstrating that the reporter gene assay worked correctly.

Keeping in mind the increased proangiogenic potential of B16-HO-1, we decided to assess the contribution of VEGF synthesis in the observed effects. To accomplish this aim the conditioned media were preincubated with anti-VEGF antibodies for 1 hour before they were transferred on HUVECs to stimulate endothelial cell proliferation. BrdU incorporation assay showed that blocking the VEGF protein in the conditioned media harvested from B16-WT or B16-HO-1 lines did not significantly influence the mitogenic response of HUVECs (Figure 6C). This suggests that VEGF is not the major factor responsible for increased angiogenic potential of melanoma cells cultured in normoxia. In contrast, augmentation of endothelial cell proliferation by conditioned media harvested from B16-WT cells incubated under hypoxic conditions was significantly reduced after treatment with anti-VEGF antibodies, indicating the importance of VEGF in mediating the hypoxia-induced angiogenesis.

Effect of HO-1 Overexpression on Transcriptome of Melanoma Cells

To determine which mediators may be involved in increased proliferation, survival, and angiogenic potential of melanoma cells overexpressing HO-1, we performed microarray screening of B16-WT and B16-HO-1 transcriptomes. Results were calculated on the basis of analyses of six different cultures of B16-HO-1 and three different cultures of B16-WT. We found that 228 of 6864 genes were differently expressed, most of them associated with intracellular transport. Among genes that could be involved in the observed effects, B16-HO-1 cell line displayed higher production of epidermal growth factor (EGF, 1.27-fold), superoxide dismutase-2 (SOD2, 1.28-fold), fibroblast growth factor receptor-1 (FGFR1, 1.37-fold), endothelial differentiation gene-7 (EDG7, 1.46-fold), metallothionein-1X (MT1X, 1.45-fold), malignant T-cell-amplified sequence-1 (MCT1, 1.5-fold), glutathione S-transferase A1 (GSTA1, 1.57-fold), hyaluronidase-1 (HYAL1, 1.79-

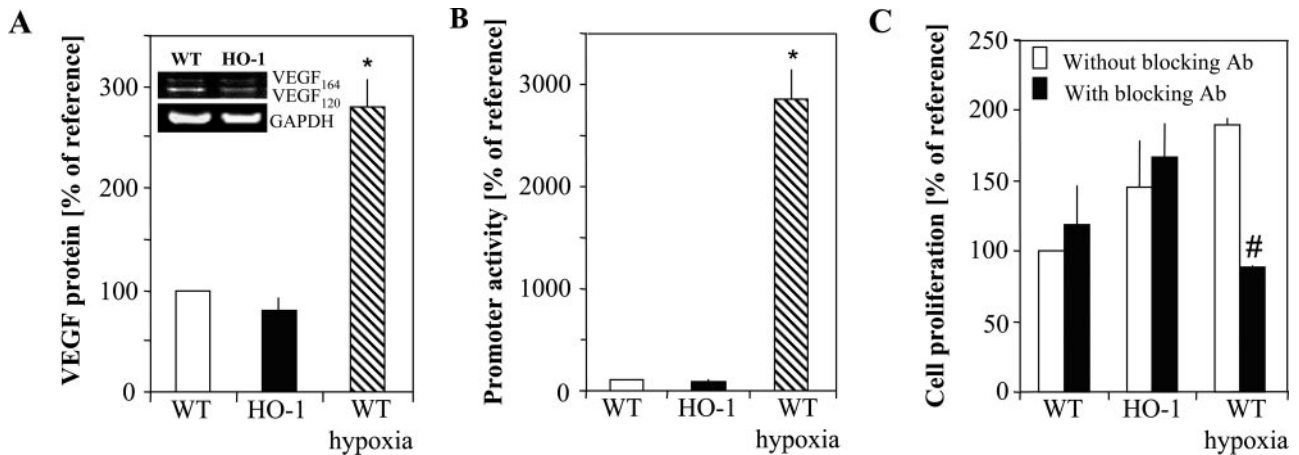


Figure 6. Effect of HO-1 overexpression on production of VEGF in melanoma cells. **A:** Concentration VEGF protein in media harvested from B16-WT and B16-HO-1 or from B16-WT cells cultured in hypoxia after overnight incubation measured by ELISA. **Inset:** Expression of VEGF mRNA demonstrated by RT-PCR. GAPDH was used as a control housekeeping gene. **B:** Activity of VEGF promoter in B16-WT and B16-HO-1 or in B16-WT cells cultured in hypoxia. Activity of promoter was measured using luciferase assay 48 hours after transfection of cells with a reporter plasmid. **C:** Effect of preincubation of conditioned media with antibodies blocking VEGF on proliferation of HUVECs. Proliferation was measured using BrdU incorporation assay 48 hours after stimulation. Each bar represents mean \pm SD of five (**A**), two (**B**), or three (**C**) experiments. Results are presented as percentage of reference value (reference, B16-WT cells). * $P < 0.05$ in comparison with B16-WT. # $P < 0.05$ in comparison with media without blocking antibodies.

fold), activating transcription factor-4 (ATF4, 1.80-fold), and thymosin- β 4 (T β 4, 1.70-fold). Among down-regulated genes were p21 (0.69-fold), B-cell translocation gene-2 (BTG2, 0.66-fold), and leukocyte-specific protein-1 (LSP1, 0.51-fold). Differences were statistically significant ($P < 0.05$).

Effect of HO-1 Overexpression on Survival Time of Mice

To investigate the effect of HO-1 overexpression on the growth of tumors, B16-WT or B16-HO-1 melanoma cells were injected subcutaneously into the back of syngeneic C57BL/6 mice (2×10^5 cells per animal). Resulting tumors were excised immediately after the death of animals to analyze tissue morphology and biochemistry. Measurement of rat HO-1 concentration by rat-specific HO-1 ELISA confirmed the expression of transgene in the tumors developed after injection of B16-HO-1 cells (amount of rat HO-1 was 5.57 ± 4.70 ng/mg of total protein; in B16-WT tumors the rat HO-1 protein was undetectable). Increased production of HO-1 in tumor cells significantly shortened the survival time of mice (Figure 7). Median survivals of animals were 38 days and 22 days after tumor cell inoculation for B16-WT and B16-HO-1 melanomas, respectively (Kaplan-Meier Wilcoxon analysis, $n = 10$, $P = 0.017$).

Effect of HO-1 on Tumor Growth

Tumors developed in all mice injected with B16-WT or B16-HO-1 melanoma cells. Their volumes were measured everyday starting at day 4 after inoculation, when the tumors became detectable under the skin, until the death of host. Surprisingly, despite differences in survivals of animals (Figure 7), the rates of tumor growth were

the same in mice bearing the wild-type and HO-1-over-expressing melanomas (Figure 8A).

Histological analyses suggested, however, that structure of B16-WT and B16-HO-1 tumors may differ. Although we did not find any significant disparity in the surface of necrotic areas (Figure 8B), density of cells in the living parts of tumors overexpressing HO-1 appeared to be greater than in the wild-type implants (Figure 8C). This observation was confirmed by Western blotting of tumor lysates with antibodies recognizing tyrosinase enzyme, a marker of melanoma cells (Figure 8D). Signals obtained from the HO-1-overexpressing tissues were much stronger than those from the same amount of the wild-type samples, indicating the higher number of melanoma cells in the B16-HO-1 tumors. It seems that the observed differences were not caused by a direct influence of HO-1 on expression of the tyrosinase because we did not find any differences in the level of

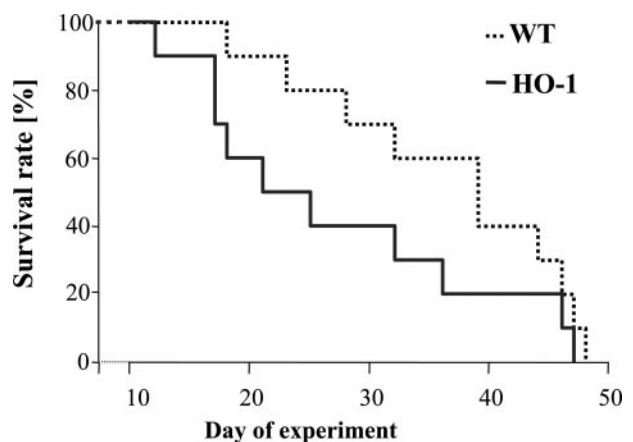


Figure 7. Survival curves of mice injected subcutaneously with B16-WT or B16-HO-1 melanoma cells (2×10^5 per animal). In each group $n = 10$, $P = 0.017$.

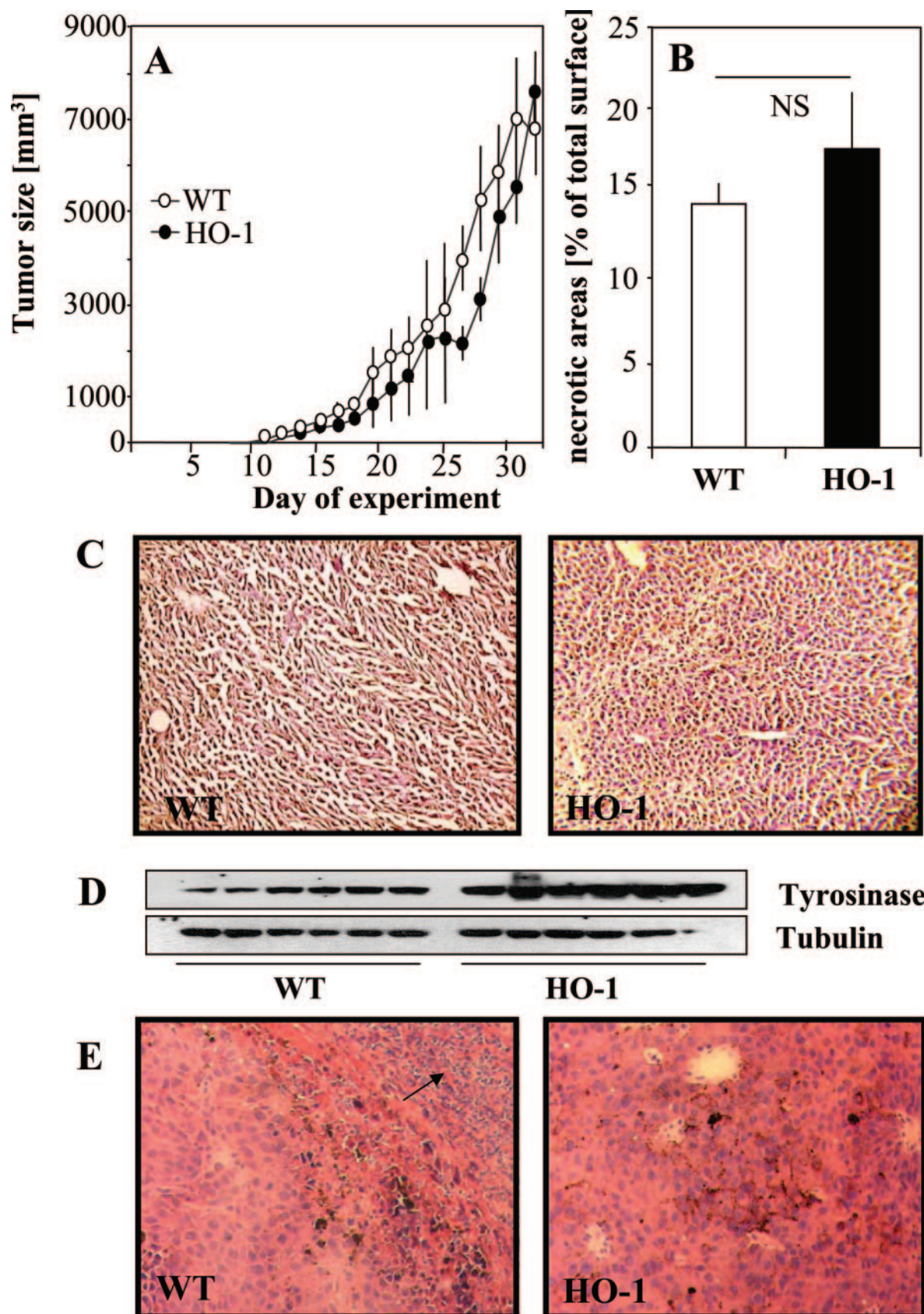


Figure 8. Morphology of tumors developed after subcutaneous injection of B16-WT and B16-HO-1 melanoma cells. **A:** Volume of tumors. **B:** Percentage of necrotic areas assessed by analysis of paraffin-embedded sections stained with H&E. **C:** Representative H&E frozen specimens showing the structure of B16-WT and B16-HO-1 tumors. **D:** Western blot showing the expression of tyrosinase in tumors. Tubulin was used as a control protein (15 μ g of total protein were loaded to each well). **E:** H&E-stained frozen specimens showing the leukocyte infiltration in B16-WT tumor (arrow). Original magnifications, $\times 200$.

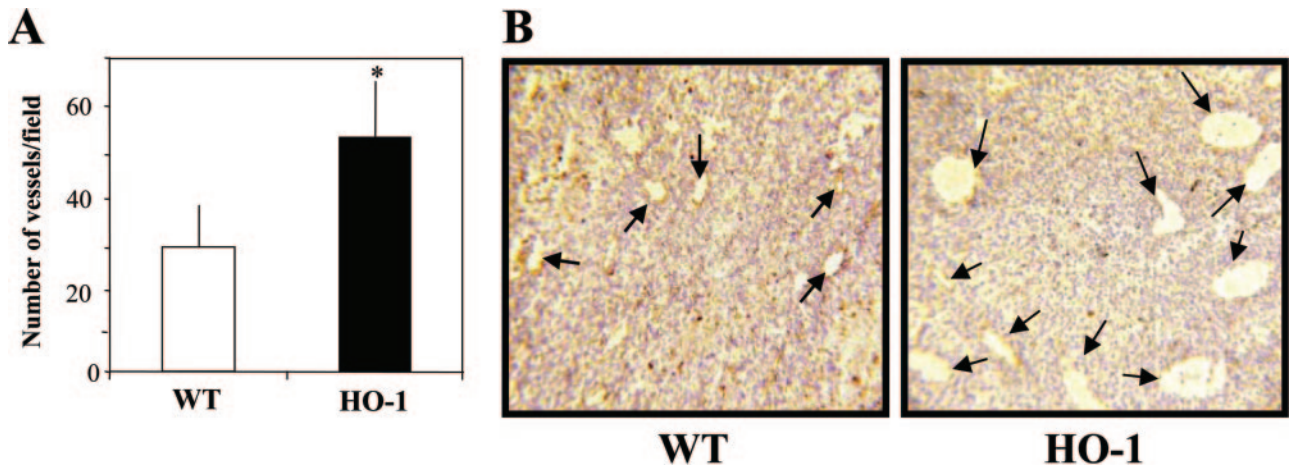


Figure 9. Effect of HO-1 overexpression on tumor vascularization. Blood vessels were stained in the frozen sections with anti-CD31 antibodies visualized by peroxidase reaction. Tissues were counterstained with hematoxylin. **A:** Representative pictures. Endothelial cells labeled by brownish reaction product are indicated by arrows. **B:** Number of vessels in sections of B16-WT and B16-HO-1 tumors counted in a blinded manner. Six specimens for each tumor were analyzed. Each bar represents mean \pm SD of 10 tumors. * $P < 0.05$ in comparison to B16-WT. Original magnifications, $\times 100$.

tyrosinase protein between B16-WT and B16-HO-1 cultured *in vitro* (data not shown). Finally, histological analysis showed that inflammatory areas and infiltrations of leukocytes appeared to be smaller in HO-1-overexpressing tumors (Figure 8E). Fluorescence-activated cell sorting analysis confirmed lower proportion of leukocytes (CD45-positive cells) in tumor cell suspensions (respectively, $24.8 \pm 7.3\%$ and $36.9 \pm 6.8\%$ in B16-HO-1 and B16-WT tumors, $P = 0.021$) and revealed that leukocyte infiltrates in both cases consisted mostly of granulocytes.

Effect of HO-1 on Tumor Angiogenesis

To assess the influence of HO-1 overexpression on the number of blood vessels, the frozen sections of tissues excised from three different tumor sites were stained immunohistochemically with primary antibodies against CD31/PECAM-1 (a marker of endothelial cells) and with secondary antibodies conjugated with peroxidase. The blood vessels were counted, in a blinded manner, by a researcher who analyzed six specimens for each tumor. As shown in Figure 9A, the number of capillaries in the tumors overexpressing HO-1 was almost twofold higher than in the wild-type melanomas (30.15 ± 9.14 and 51.08 ± 13.05 vessels per microscopic field for B16-WT and B16-HO-1 tumors, respectively). Moreover, the diameter of vessels in tissues with HO-1 overexpression seemed to be bigger than that in B16-WT implants (Figure 9B).

Effect of HO-1 on Expression of Angiogenic and Inflammatory Cytokines

Because HO-1 is regarded as an anti-inflammatory enzyme, we decided to investigate the effect of HO-1 overexpression in the tumors on generation of inflammatory mediators. In addition, we evaluated the level of VEGF. Concentrations of cytokines were measured in the serum

of control or melanoma-bearing mice and in the tissue lysates prepared from B16-WT and B16-HO-1 tumors. We demonstrated that in the blood of mice that developed tumors the levels of VEGF were elevated when compared with healthy animals (Figure 10A). Furthermore, the mean concentration of VEGF in serum of B16-

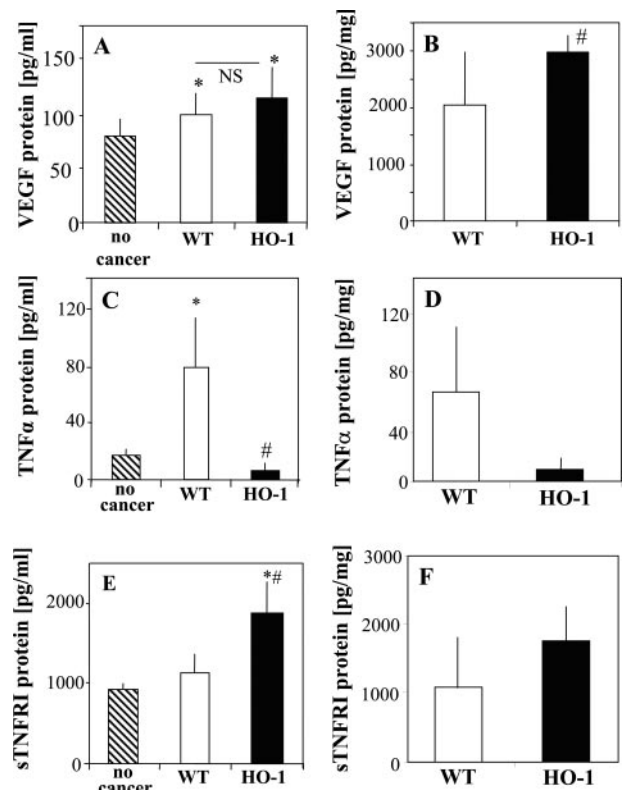


Figure 10. Concentration of VEGF, TNF, and sTNF-RI proteins in plasma of mice bearing B16-WT or B16-HO-1 tumors and in cell lysate prepared from the tumor tissues. Shown are VEGF in plasma (**A**), VEGF in tumor lysates (**B**), TNF in plasma (**C**), TNF in tumor lysates (**D**), sTNF-RI in plasma (**E**), and sTNF-RI in tumor lysates (**F**). Each bar represents the mean \pm SD of six samples. * $P < 0.05$ in comparison to healthy animals; # $P < 0.05$ in comparison to B16-WT animals.

HO-1-injected animals was slightly higher than in B16-WT-inoculated mice, but the difference was not statistically significant ($P > 0.1$). This tendency, however, was more pronounced in the tumor lysates, where concentrations of VEGF protein in B16-HO-1 samples were significantly higher ($P < 0.05$) than in B16-WT tumors (Figure 10B).

To investigate the production of inflammatory mediators, we analyzed the serum of healthy or tumor-harboring mice using a cytokine antibody array for 22 proteins. The obtained results suggested that two of the cytokines studied, that is, TNF and its soluble receptor sTNF-RI, were differently expressed in mice bearing B16-WT and B16-HO-1 tumors. These preliminary analyses were then verified by quantitative ELISA performed both in sera of mice and in lysates prepared from tumors. We found that mean concentration of TNF in animals bearing B16-WT melanoma was significantly elevated (approximately fourfold) above the level typical for healthy mice (Figure 10C). In contrast, no such increase was detected for melanoma cells overexpressing HO-1. In these animals injected with B16-HO-1 cells, the level of TNF in the blood was barely detectable, similar to the healthy mice.

The opposite relation was demonstrated for sTNF-RI. We did not find any significant differences between healthy mice and those inoculated with B16-WT, whereas stronger productions of sTNF-RI were observed in mice bearing the B16-HO-1 tumors (Figure 10E). Similar trends, namely the decreased synthesis of TNF and increased release of sTNF-RI, were also demonstrated in the lysates prepared from B16-HO-1 tumor tissues (Figure 10, D and F). Because of larger variation between the samples, these tendencies did not reach statistical significances. Of note, melanoma cells seem not to be a source of TNF because this protein was not detectable in media harvested from the cultured B16-WT or B16-HO-1 lines (data not shown).

Effect of HO-1 Overexpression on Metastatic Potential of Melanoma

B16(F10) cells used in our experiments did not produce visible metastases when injected subcutaneously. Therefore, to assess their ability of homing and growing in distant organs, we injected B16-WT and B16-HO-1 cell lines into the tail vein of mice (2×10^5 cells/per animal). Such treatment resulted in formation of tumors within the lungs. We compared the number of metastases at days 7, 14, and 21 after inoculation. As shown in Figure 11, overexpression of HO-1 strongly increased number and size of tumors. The difference was visible already on day 7 (mean number of visible metastases was 7.2 ± 4.5 and 21.9 ± 20.8 for B16-WT and B16-HO1, respectively; $P = 0.054$) and was even more pronounced at the later time points (on day 14: 8.0 ± 7.8 for B16-WT and 33.2 ± 23.8 for B16-HO-1, $P = 0.008$; on day 21: 19.3 ± 12.7 for B16-WT and 51.8 ± 24.7 for B16-HO-1, $P = 0.004$). These data suggest that increased number of metastases and/or their faster growth can contribute to the

shorten survival of animals bearing melanoma tumors that overexpress HO-1.

Discussion

HO-1 is an inducible enzyme whose expression is often increased in tumors, especially those subjected to chemotherapy or photodynamic therapy.^{15,32} The consequences of its up-regulation are, however, not well recognized. Here we investigated the effects of HO-1 overexpression in murine and human melanoma cells. The most important findings of our study are that 1) overexpression of HO-1 augments the proliferation, improves the resistance to oxidative stress, and increases angiogenic potential of murine and human melanoma cells; 2) increased production of HO-1 in B16(F10) tumor cells shortens survival time of mice, results in development of more packed tumors, with higher number of cells, reduced inflammatory edemas, and increased vascularization, and leads to augmented metastatic potential of melanoma; and 3) mice harboring melanomas overexpressing HO-1 have lower levels of TNF and higher concentrations of sTNF-RI in the serum than mice bearing wild-type melanoma.

Many conclusions concerning the role of HO-1 in regulation of cell activities are driven by experiments performed using its pharmacological activators (eg, heme, CoPPIX, CoCl_2) and inhibitors (eg, SnPPIX, ZnPPIX, ZnMPPIX, ZnDPPIX). It must be kept in mind, however, that all these compounds display strong, unspecific effects.²⁰ For example, exposure of Hep3B cells to heme, CoCl_2 , and SnPPIX resulted in a potent HO-1-independent induction of erythropoietin.³⁴ In THP-1 monocytes heme, ZnMPPIX, ZnDPPIX, and CoPPIX, regardless of their effects on HO-1, reduced the transduction of interferon- γ signal, whereas SnPPIX enhanced it and elevated expression of MHC-II.³⁵ Of importance, it has been shown that CoPPIX, SnPPIX, and ZnPPIX are direct inhibitors of caspase-3 and caspase-8, and thereby, they decrease the rate of apoptosis, independently of the HO-1 pathway.³⁶ Taken together, these results indicate that pharmacological modulators of HO-1 activity are not suitable tools to investigate the effects of HO-1, at least in studies on inflammation and cell viability.

Likewise, *in vivo* experiments have demonstrated that protoporphyrins significantly decreased tumor blood flow in rats, but this effect was apparently independent of HO-1 because both ZnPPIX (HO-1 inhibitor) and CuPPIX (compound that does not modulate HO-1 activity) were similarly efficient.^{37,38} Finally, even a relatively high dose of ZnPPIX ($45 \mu\text{mol/kg}$) injected intraperitoneally into the rats may be unable to inhibit the activity of HO-1 in tumors, although it could produce HO-1-independent effects.³⁷ Therefore, to avoid the drawbacks of pharmacological modulators of HO-1, we decided to use genetically modified melanoma cell lines, transduced with adenoviral vectors or stably transfected with HO-1 expression plasmid. Such cells displayed high HO-1 expression as shown at both the mRNA and protein levels

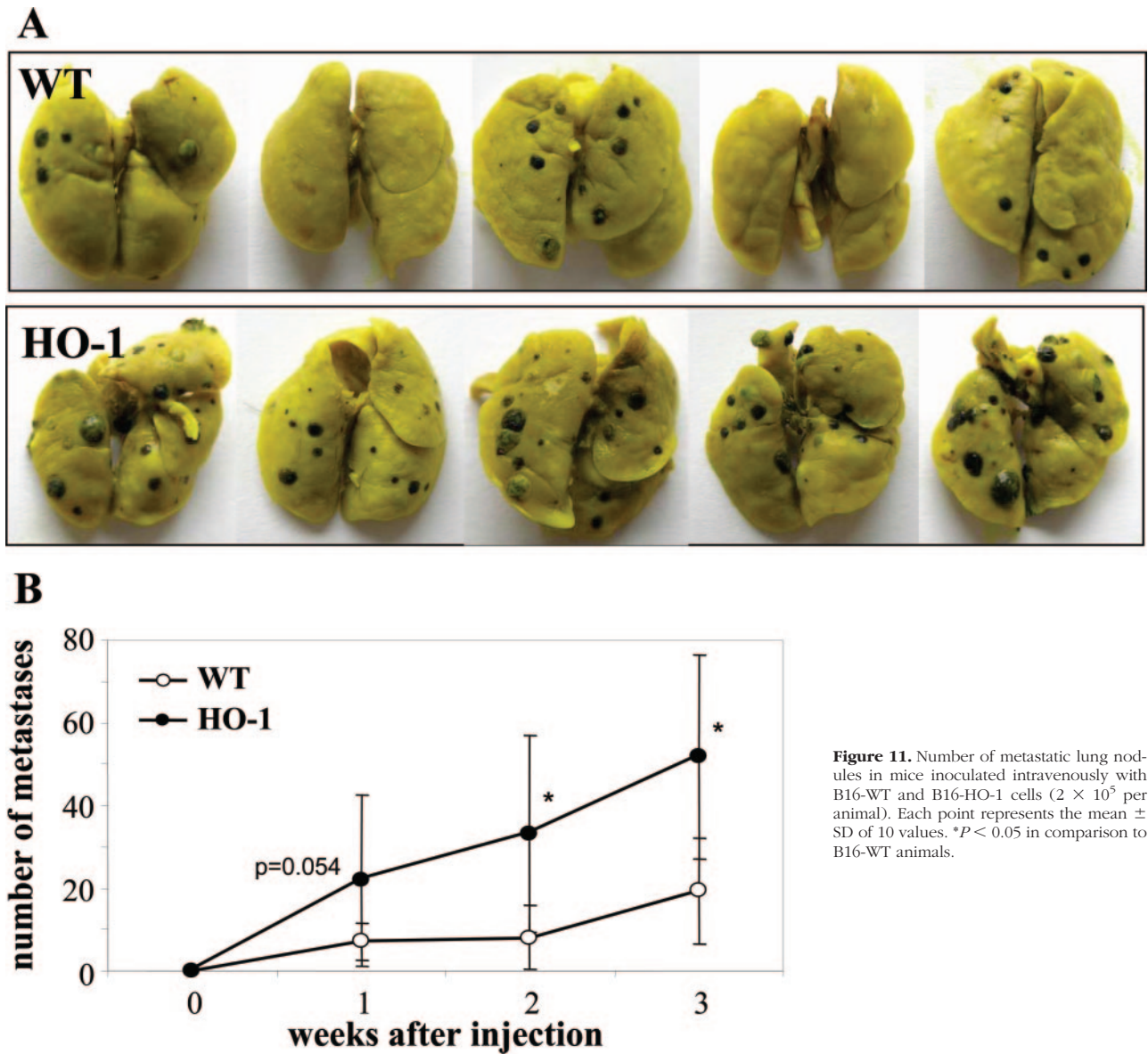


Figure 11. Number of metastatic lung nodules in mice inoculated intravenously with B16-WT and B16-HO-1 cells (2×10^5 per animal). Each point represents the mean \pm SD of 10 values. * $P < 0.05$ in comparison to B16-WT animals.

and could be directly compared with the wild-type counterparts.

Strong expression of HO-1 is often observed in tissues with a high rate of cell proliferation, such as epidermis during cutaneous wound healing,³⁹ psoriatic skin lesions,¹¹ or tumors including adenocarcinoma, hepatoma, sarcoma, glioblastoma, melanoma, and squamous carcinoma.^{14,40–42} Contradictory results published so far indicate that the role of HO-1 in regulation of cell cycle is strongly tissue-type-dependent. In some models, for example in astroglia, pulmonary or proximal tubular epithelium, vascular smooth muscles, or breast carcinoma, activation of HO-1 results in cell growth arrest,^{14,40–43} and this effect can be associated with up-regulation of the cyclin-dependent kinase inhibitor p21.⁴³ In the other cell types, such as epidermal keratinocytes and vascular endothelium, pharmacological induction or genetic overexpression of HO-1 stimulates cell division.^{10,12,44} Increased proliferation is accompanied by down-regulation

of p21 and higher expression of EGF, a potent mitogen.⁴⁴ Of importance, it has been reported recently that targeted knock-down of HO-1 in the pancreatic cancer cells led to growth inhibition suggesting the stimulatory effect of HO-1.¹⁵

Our results demonstrate for the first time that HO-1 activity increases proliferation in melanoma. Microarray analysis suggests that it can be associated with lower expression of two negative regulators of cell cycle, p21 and BTG2. Importance of p21 down-regulation in cutaneous malignant melanoma progression has been already indicated in clinical studies,⁴⁵ whereas inhibition of BTG2, an inhibitor of pRb functions and G₁/S transition, was postulated as an important step in renal cancer development.⁴⁶ In addition, B16-HO-1 cell line produces more EGF and has a stronger expression of two mitogenic receptors: EDG7, a specific receptor for lysophosphatidic acid, and FGFR1, a receptor for fibroblast growth factors. Earlier experiments done in ovarian can-

cer and melanoma cells have shown that up-regulation of EDG7 or FGFR1 may increase proliferation.^{47,48}

HO-1 is also known as a prominent antioxidative enzyme. Its importance in protection against free radicals is illustrated by disturbed development of HO-1 knockout mice. In response to chronic hypoxia, they exhibit enhanced lipid peroxidation and accentuated oxidative damages. In addition, cultured fibroblasts from HO-1 knockout mice are extremely susceptible to oxidative stress and produce high amounts of reactive oxygen species.⁴⁹ Accordingly, in the sole human patient characterized, the HO-1 deficiency resulted in the sustained oxidative stress and augmented inflammatory reactions, which led to severe endothelial damage.⁵⁰

Our data suggest that B16(F10) melanoma cells are relatively sensitive to oxidative insults because ~50% of cells die after a 4-hour exposure to 100 $\mu\text{mol/L}$ H_2O_2 . Much better survival under the same conditions was observed for keratinocytes, fibroblasts, or even endothelial cells (data not shown). This result is not surprising because it is known that expressions of antioxidative enzymes such as catalase, glutathione peroxidase, and superoxide dismutase, are reduced in a range of tumors.^{51–53} In addition, in melanoma the transformation causes damage of melanosomes and thereby enhances reactive oxygen species production, increasing vulnerability of cells to oxidative stress.⁵⁴ However, two other melanoma cell lines studied (S91 and Sk-mel188) were much more resistant to treatment with H_2O_2 , indicating that sensitivity to oxidative stress is cell-type-dependent.

Numerous experiments have confirmed that HO-1 induction provides a cellular protection, as first demonstrated in endothelium⁵⁵ and then in many other cell types, including fibroblasts, renal proximal tubule cells, and islet cells.^{1,8} Furthermore, similar abilities of HO-1 were described in animal models of brain, heart, kidney, lung, and liver failures.^{2–6}

Defensive effects of HO-1 were also found in tumors. Gastric cancer cell lines with up-regulated HO-1 expression showed resistance to apoptotic stimuli, which was related to increased caspase inhibitory protein-2 (c-IAP2) and decreased caspase-3 activities.⁵⁶ Moreover, on the basis of experiments with HO-1 inhibitors, Fang and co-workers⁵⁷ proposed that HO-1 protected hepatoma, colon carcinoma, and sarcoma solid tumor cells against oxidative stress and decreased their apoptosis. Some opposite observations have also been reported because overexpression of HO-1 increased oxidative damage to mitochondria and resulted in augmented apoptosis in breast carcinoma and astroglia.^{14,42} In our study, however, melanoma cells overexpressing HO-1 were significantly more resistant to H_2O_2 -induced oxidative stress than the wild-type ones because viability of B16-HO-1 after a 4-hour exposure to 200 $\mu\text{mol/L}$ H_2O_2 was almost threefold higher than that of B16-WT. Analysis of the transcriptome suggests that the cytoprotective effect of HO-1 in B16-HO-1 cells can be associated with up-regulation of ATF4, a transcription factor that interacts with Nrf2 and increases production of enzymes with antioxidant and xenobiotic detoxification functions.⁵⁸ We also found a higher expression of MT1X, SOD2, and GSTA1,

which are known to enhance cancer resistance to radiotherapy or chemotherapy.^{59–61} In addition, metallothionein MT1X can be directly involved in protection against H_2O_2 .⁶²

We also demonstrated that overexpression of HO-1 in melanoma cells potentially increases their metastatic potential. B16(F10) cell line injected subcutaneously does not produce metastases that could be macroscopically detected as nodules in the lungs; therefore, we used an intravenous inoculation model. Animals treated with B16-HO-1 developed fourfold more and clearly bigger nodules than their counterparts injected with B16-WT. Likewise, increased occurrence of lung metastases has been described in the rats bearing pancreatic cancer implants overexpressing HO-1,¹⁶ suggesting that prometastatic action of HO-1 is a more general event.

Data obtained from microarrays suggest that formation of metastases by B16-HO-1 is associated with an increased level of T β 4. This small protein is known to be up-regulated in metastatic nodules, and its expression correlates with metastatic potential of tumors, mostly by activating cell migration and stimulating angiogenesis.⁶³ Interestingly, in an experimental model very similar to ours, T β 4-overexpressing B16(F10) melanoma cells injected intravenously to mice produced almost fivefold more metastatic lung nodules than the wild-type cells.⁶³ This result resembles very much that observed after HO-1 overexpression. Another prometastatic gene up-regulated in B16-HO-1 is the hyaluronidase HYAL1, which degrades hyaluronic acid, involved in regulation of cell adhesion and migration, to small proangiogenic fragments. Overexpression of HYAL1 in bladder cancer cells increases their invasiveness.⁶⁴ One can expect that a similar effect could exist in melanoma.

It seems that cytoprotection, increased proliferation, and augmented metastatic potential provided by overexpression of HO-1 may be beneficial for development of tumor tissue but detrimental for tumor-bearing animals. Our *in vivo* experiment confirms this supposition. Animals inoculated subcutaneously with B16-WT survived longer (median value, 38 days) than their counterparts inoculated with B16-HO-1 cells (median value, 22 days). To the best of our knowledge, this is the first demonstration that a different level of HO-1 in the tumor may influence the survival of the hosts. Of note, a very recent report confirmed the importance of HO-1 expression level in development of melanoma in humans. Analysis of HO-1 promoter polymorphism in healthy people and cancer patients indicates that the risk of acquiring and more serious progression of malignant melanoma is significantly higher in people harboring a shorter (GT) n repeats sequence, associated with a higher HO-1 activity.⁶⁵

It must be stressed, however, that effects of HO-1 activation may be different in the case of tumor induction compared with tumor progression. Two clinical trials analyzing the frequency of tumor development (oral squamous cell carcinoma in betel chewers and lung adenocarcinoma in cigarette smokers) have suggested that high expression of HO-1 may be beneficial for patients and may decrease the incidence of carcinogenesis. It was demonstrated that frequencies of cancers are lower

in patients with a shorter sequence of (GT)_n in the HO-1 promoter that are supposed to have a higher level of HO-1 induction in response to oxidative stress.^{66,67} It seems that these benefits may result from antioxidative properties of HO-1, which contribute to protection of healthy cells against carcinogens. However, in already existing tumors, the increased level of HO-1 leads to better tumor cell survival and faster proliferation.⁶⁸

Accordingly, it has been shown that cancer cells treated with HO-1 inhibitors become vulnerable to insults caused by various cytotoxic agents, especially camptothecin and doxorubicin.⁶⁹ There are also several reports describing the influence of HO-1 on tumor sizes. For example, intra-arterial or intraperitoneal injection of Zn-PPIX, SnMPPIX, or PEG-ZnPPIX suppressed the enlargement of solid hepatoma, pancreatic tumors, or sarcoma, confirming the stimulatory role of HO-1 in the tumor growth.^{16,25,69,70}

To our surprise, despite the shorter survival time of mice bearing the HO-1-overexpressing melanoma, we did not find any differences in the tumor sizes after subcutaneous inoculation of B16-HO-1 and B16-WT cells. However, the histological analysis showed that melanomas overexpressing HO-1 formed more packed tumors with a higher cell density. This observation is supported by a higher concentration of tyrosinase in the cell lysates prepared from B16-HO-1 tissue. In addition, we noticed an augmented edema within the wild type but not within the HO-1-overexpressing tumors. Thus, we suppose that overexpression of HO-1 may be associated with increased tumor growth, but because of the different structure of tissue, we were not able to find differences in volumes of subcutaneously inoculated tumors.

Apart from the antioxidative properties, HO-1 is also considered as a proangiogenic enzyme. Its ability to stimulate synthesis of VEGF was evidenced in cultured vascular smooth muscles,¹⁸ microvascular endothelium,¹² and keratinocytes.²¹ Likewise, in the present experiments we showed that overexpression of HO-1 increased angiogenic potential of melanoma cells because conditioned medium harvested from B16-HO-1 were stronger inducers of endothelial proliferation and formation of capillaries than those from B16-WT. However, this was not associated with up-regulation of VEGF. We demonstrated that, in contrast to many other tissues studied, overexpression of HO-1 in melanoma cells did not influence the production of VEGF protein or mRNA or activity of the promoter. Accordingly, induction of angiogenic response of endothelial cells was not inhibited by preincubation of conditioned media with anti-VEGF neutralizing antibodies. Thus, the ability of HO-1 to increase angiogenic response is mediated through some other factors.

We hypothesize that the primary candidates for such mediators can be EGF, T β 4, HYAL1, and MCT1, all up-regulated in B16-HO-1, as detected by microarray analysis. It has been demonstrated that elevated expression of EGF, a mitogen acting on endothelial cells among others, augments angiogenesis and enhances metastasis formation in melanoma.⁷¹ T β 4 has a potent chemotactic properties for endothelial cells,⁷² and its expres-

sion was associated with increased vascularization of B16(F10) tumors.⁶³ Likewise, overexpression of HYAL1 and MCT1 resulted in higher microvessel density in bladder cancer⁶⁴ and breast cancer,⁷³ respectively. Confirmation of the real involvement of all those proteins in HO-1-induced angiogenesis, however, requires additional studies.

Increased angiogenic potential of B16-HO-1 melanoma was also demonstrated *in vivo*. We found that overexpression of HO-1 leads to a stronger vascularization of tumor tissue. Similar observations have been also reported for gliomas, pancreatic cancer, and vertical growth melanomas.^{16,23,28} Furthermore, we noticed that the vessels in tumors derived from B16-HO-1 had a bigger diameter than those in the wild-type implants. Interestingly, in the tumors overexpressing HO-1 and in the plasma of B16-HO-1-injected mice, we observed the increased VEGF protein concentrations. However, keeping in mind the lack of effect of HO-1 on VEGF production in melanoma cells *in vitro*, we suppose that influence is indirect *in vivo*.

Tumorigenesis is associated with inflammation, although the exact role of inflammatory reactions in tumor growth may be different. Also in the present study, we found the increased synthesis of TNF in mice injected with melanoma cells. Numerous reports have shown that activation of HO-1 diminishes inflammation and may result in immunosuppression,⁷⁴ the effects mediated mostly by production of CO and/or bilirubin.⁷⁵ The importance of HO-1 is elegantly illustrated by study in HO-1^{-/-} mice, in which HO-1 deficiency results in strongly increased generation of proinflammatory cytokines, including TNF, and in general, Th1 shift in immune activity.⁷⁶ Moreover, constitutive expression of HO-1 observed in CD4⁺CD25⁺ regulatory T cells indicates its crucial role in modulation of immune response.⁷⁷ Accordingly, elevated HO-1 expression may lead to clonal deletion of CD4⁺ T cells, resulting in a specific immunomodulation and in prolongation of transplanted organ survival.⁷⁸ Because the important mediator of HO-1 anti-inflammatory activity is CO, which can diffuse through cell membrane, one can suppose that overexpression of HO-1 in melanoma cells can directly modulate activity of infiltrating immune cells, eg, their proliferation, motility, and production of proinflammatory mediators. For example, exposure of macrophages to CO can result in decreased synthesis of cytokines, including TNF.⁷⁵

Accordingly, our study demonstrates that overexpression of HO-1 in melanoma cells inhibits production of TNF by the tumor-bearing mice. On the other hand, a concentration of soluble TNF receptor (sTNF-RI) is significantly higher in the blood of animals injected with B16-HO-1. Thus, HO-1 inhibits the TNF pathway not only by down-regulating expression of this cytokine but also by increasing the availability of TNF scavenger. Likewise, as in the case of VEGF, the effect of HO-1 is indirect because melanoma cells cultured *in vitro* did not produce measurable amounts of TNF (ELISA, data not shown). Finally, it seems that stronger HO-1 activity in melanoma cells reduces inflammatory edema and leukocyte infiltration. We found that B16-HO-1 displays a lower expression of

LPS1, an intracellular molecule involved in leukocyte migration and recruitment to sites of inflammation.⁷⁹ In addition, the up-regulated synthesis of T β 4 can be accompanied by a decrease in inflammatory reaction.⁴⁴ The exact role of these molecules in regulation of leukocyte infiltrates in melanoma tumors remains to be established.

Investigations of the role of HO-1 seem to be important not only for better understanding of tumor growth regulation but also for clinical practice. Although some reports describe a selective diminishing of HO-1 in malignant cancer cells such as adenocarcinoma or tongue squamous carcinoma,^{80,81} the majority of analyses indicate that expression of HO-1 is strongly up-regulated in various tumors, including adenocarcinoma, lung carcinoma, hepatoma, sarcoma, glioblastoma, melanoma, squamous carcinoma, or pancreatic and prostate cancers.^{15,24–29} In some neoplastic cells the expression of HO-1 becomes constitutive as in chronic myeloid leukemia, in which BCR/ABL kinase activates HO-1 promoter.⁸²

Importantly, treatment of cancer cells with chemotherapeutics such as gemcitabine or *cis*-platin as well as their exposure to radiation or photodynamic therapy can further increase HO-1 expression.^{15,31,32} This up-regulation in response to therapeutic procedures may have important consequences. For example, in chronic myeloid leukemia-derived cell line K562, induction of HO-1 was found to counteract Gleevec (STI571)-induced apoptosis.⁸² Likewise, our recent results have shown that increased expression of HO-1 in mice harboring adenocarcinoma and treated with photodynamic therapy results in much faster regrowth of tumors.³² Thus, our results support the idea that HO-1 may be a potential target in anti-tumor therapy. Likewise, as it was suggested for chronic myeloid leukemia,⁸² colon carcinoma,⁶⁹ adenocarcinoma,³² or pancreatic cancer,¹⁵ pharmacological inhibition of HO-1 may be a new option in treatment of melanoma and may be used as sensitizer to chemotherapy and radiotherapy.

In conclusion, we showed that HO-1 overexpression potentiates melanoma cancer aggressiveness, by increasing tumor cell proliferation, resistance against oxidative stress, and augmentation of angiogenic and metastatic potentials both *in vitro* and *in vivo*. In consequence, increased activity of HO-1 in tumor cells results in shorter survival of tumor-bearing mice. Therefore, we suppose that down-regulation of HO-1 might be beneficial in therapy of melanoma.

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